

Research Article

Genetic Polymorphisms in Genes Related to Oxidative Stress (*GSTP1*, *GSTM1*, *GSTT1*, *CAT*, *MnSOD*, *MPO*, *eNOS*) and Survival of Rectal Cancer Patients after Radiotherapy

Silvia Funke,¹ Angela Risch,² Alexandra Nieters,¹ Michael Hoffmeister,³ Christa Stegmaier,⁴ Christoph M. Seiler,⁵ Hermann Brenner,³ and Jenny Chang-Claude¹

¹ Division of Cancer Epidemiology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

² Division of Epigenomics and Cancer Risk Factors, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

³ Division of Clinical Epidemiology and Aging Research, German Cancer Research Center, Bergheimer Str. 20, 69115 Heidelberg, Germany

⁴ Saarland Cancer Registry, Praesident-Baltz-Str. 5, 66119 Saarbruecken, Germany

⁵ Department of General, Visceral and Transplantation Surgery, University of Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany

Correspondence should be addressed to Jenny Chang-Claude, j.chang-claude@dkfz.de

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Radiotherapy exerts part of its antineoplastic effect by generating oxidative stress, therefore genetic variation in oxidative stress-related enzymes may influence survival of rectal cancer patients. We hypothesized that genetic polymorphisms associated with higher amounts of reactive oxygen species (ROS) that exaggerate cytotoxic activity could improve survival after radiotherapy. We followed 114 rectal cancer patients who received radiotherapy for an average of 42.5 months. Associations between genotypes (*GSTP1*, *GSTM1*, *GSTT1*, *CAT*, *MnSOD*, *MPO* and *eNOS*) and overall survival were assessed using Kaplan-Meier curves and Cox proportional hazards regression. As hypothesized, patients carrying low ROS producing *eNOS* Glu298Asp asparagine allele showed an increased hazard of death compared to homozygous carriers of the glutamine allele (hazard ratio (HR): 2.10, 95% confidence interval (CI): 1.01–4.38). However, carriers of low ROS producing *MPO* G463A A allele had a decreased hazard of death compared to patients homozygous for the G allele (HR: 0.44, 95% CI: 0.21–0.93) although patients homozygous for the A allele had a slightly increased hazard (HR: 1.12, 95% CI: 0.25–5.08). This explorative study provides first results and highlights the need for further, larger studies to investigate association between genetic variation in oxidative stress genes and survival of rectal cancer patients who received radiotherapy.

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1. Introduction

Radiotherapy is the current neoadjuvant and adjuvant treatment standard for rectal cancer in addition to surgery [1]. In combination with chemotherapy, radiation showed effectiveness in tumor downstaging and has facilitated sphincter-saving surgery in the neoadjuvant setting [2]. Radiation exerts part of its cytotoxic effects through indirect action by producing reactive oxygen species (ROS) [3]. This oxidative damaging potential is utilized to treat cancer by eradicating the malignant cells through massive cellular damage to

macromolecules or indirectly through triggering abnormal signaling and cell cycle regulation [4]. Individual variability in enzymes participating in cell defense mechanisms against ROS generated by radiation may account for individual differences in prognosis.

Enzymes involved in ROS neutralizing pathways include glutathione-S-transferases (GSTs), manganese superoxide dismutase (MnSOD), and catalase (CAT). GSTs participate in carcinogen metabolism and have been related to detoxification of products generated by UV radiation-induced oxidative stress [5]. Furthermore, they are known to

inhibit the mitogen-activated protein (MAP) kinase pathway through which apoptosis, stimulated by oxidative or chemical stress, is induced. By direct binding to c-Jun-*N*-terminal kinase 1 that activates the MAP kinase system, GSTs participate in the metabolism of lipids and DNA products derived from oxidative stress [6]. Polymorphisms in GST genes include the *GSTP1* Ile¹⁰⁵Val polymorphism and the deletion polymorphisms of *GSTM1* or *GSTT1* leading to diminished or abolished enzyme activity [7, 8]. MnSOD plays a crucial role in endogenous defense mechanisms against ROS, by converting superoxide radicals to H₂O₂ [9]. If not subsequently removed by other antioxidant defense enzymes, H₂O₂ itself can contribute to further generation of ROS catalyzed by myeloperoxidase (MPO). The alanine allele of *MnSOD* Val⁹Ala polymorphism has been associated with overexpression of MnSOD, resulting in an increased production of H₂O₂, which has been proposed to generate increased levels of ROS, if not subsequently neutralized [10]. CAT participates in defense mechanisms against oxidative stress by controlling the intracellular concentration of H₂O₂ by conversion into H₂O and O₂ [11]. The variant T allele of CAT C262T polymorphism has been associated with lower enzyme activity compared to the C allele and, thus, increased levels of ROS [12, 13].

Enzymes such as MPO and endothelial nitric oxide synthase (eNOS) are involved in the generation of ROS. MPO catalyzes a reaction between H₂O₂ and chloride to generate hypochlorous acid, a potent oxidizing agent [14]. The G463A single nucleotide polymorphism (SNP) shows a decreased affinity of the variant A allele to the SP1 binding site, resulting in a lower transcriptional activation. The A allele has therefore been suggested to be associated with lower levels of ROS [15]. eNOS generates both ROS and NO radicals. NO has complex cellular effects, either cytoprotective or cytotoxic depending on its concentration [16, 17]. The asparagine allele of *eNOS* Glu²⁹⁸Asp polymorphism has been associated with reduced levels of NO and ROS [18].

So far, the effect of genetic variants in oxidative stress genes on survival in rectal cancer patients who received radiation therapy has not been assessed. However, studies in breast cancer patients have observed a survival benefit for patients with high ROS level genotypes [10, 19]. We hypothesized that polymorphisms in *GSTP1*, *GSTM1*, *GSTT1*, *CAT*, *MnSOD*, *MPO*, and *eNOS* associated with high ROS levels would prolong overall survival in rectal cancer patients who received radiotherapy and used data of two population based studies to evaluate this.

2. Material and Methods

2.1. Patient Population and Data Collection. This followup study included 473 patients with newly diagnosed, histologically proven, invasive rectal cancer recruited in a population-based case-control study (DACHS) ($n = 237$) and a population-based patient study (ESTHER II) ($n = 236$). Baseline inclusion criteria of the DACHS study were residence in the Rhein-Neckar-Odenwald region, diagnosis between January 2003 and December 2004, age of 30 years or older. Patients were eligible to participate in the

ESTHER II study if they were residents of the Saarland, diagnosed between January 2001 and March 2003, and aged between 50 and 74 years. Further eligibility criteria for both studies were sufficient knowledge of the German language to participate in a personal interview and availability of a blood (DACHS/ESTHER II) or a mouthwash (DACHS) sample. The studies were approved by the corresponding ethics committees (Medical University of Heidelberg, State Medical Boards of Baden-Wuerttemberg, Rhineland-Palatinate, and Saarland, resp.). Written informed consent was obtained from all study participants.

After an average of three years after diagnosis detailed information on rectal cancer treatment was collected from treating physicians. Data on vital status, date of death, and cause of death were provided by registration offices in the Rhein-Neckar-Odenwald region and by the Saarland Cancer Registry, respectively. Treatment information was available for 73% ($n = 346$) of the patients. Patients which provided treatment information and those without treatment information did not differ with respect to sex, age distribution, stage of disease, vital status, and cause of death.

Altogether, 132 patients received radiotherapy. The present analysis excluded patients lacking information on vital status ($n = 1$), date of death ($n = 1$), and start of radiation treatment ($n = 15$). In addition, patients with a survival time below one month were excluded from analysis ($n = 1$). Altogether, 114 patients (DACHS: $n = 58$, ESTHER II: $n = 56$) were eligible for analysis.

2.2. Genotyping. Genomic DNA was isolated from blood using the FlexiGene DNA Kit (Qiagen GmbH, Hilden, Germany) (DACHS) or from EDTA anticoagulated blood using a previously described alcohol-precipitation based method (ESTHER II) [20]. A 10% random sample was genotyped twice for quality control.

GSTP1 Ile¹⁰⁵Val polymorphism (rs1695) was analyzed using fluorescence-based melting curve analysis as described previously [21]. Homozygous deletions of *GSTM1* and *GSTT1* were assessed using a multiplex PCR followed by gel electrophoresis as described previously [22]. SNPs in *CAT* (rs1001179), *MnSOD* (rs4880), *MPO* (rs2333227), and *eNOS* (rs1799983) were analyzed using polymerase chain reaction (PCR) followed by Pyrosequencing technology (Biotage, Uppsala, Sweden). Detailed information on primers and cycling conditions of Pyrosequencing analyses is provided in the supplements.

2.3. Statistical Analyses. Analysis was performed using SAS software, version 9.1 (SAS Institute, Cary, NC, USA). All statistical tests were two sided with a significance level of $\alpha = 0.05$. Baseline characteristics between deceased and censored patients were compared using *t*-test for normally distributed continuous variables and χ^2 -test for categorical variables. For SNPs in *GSTP1*, *CAT*, *MnSOD*, *MPO*, and *eNOS* genotype data were categorized in three categories (homozygous wild-type allele (reference), heterozygous allele carriers, homozygous variant allele carriers) as well as two categories (carrier status versus noncarrier). Deletion polymorphisms in

TABLE 1: Descriptive characteristics of deceased and censored patients.

	All (N = 114)		Deceased (N = 35)		Censored (N = 79)		P
	N	%	N	%	N	%	
Sex							.65*
female	36	31.6	10	28.6	26	32.9	
male	78	68.4	25	71.4	53	67.1	
Age (years), mean			62.1		63.3		.13 [†]
Age groups (years)							.16*
<49	6	5.2	4	11.4	2	2.5	
50–59	19	16.7	3	8.6	16	20.3	
60–69	70	61.4	21	60.0	49	62.0	
70–79	18	15.8	7	20.0	11	13.9	
>80	1	0.9	0	0.0	1	1.3	
BMI (kg/m ²), mean			26.3		26.0		1.00 [†]
BMI groups (kg/m ²)							.96*
<25	49	43.0	15	42.9	34	43.0	
25–29.9	47	41.2	14	40.0	33	41.8	
≥30	18	15.8	6	17.1	12	15.2	
Stage							<.01*
1	10	8.8	1	2.9	9	11.4	
2	34	29.8	5	14.3	29	36.7	
3	58	50.9	19	54.3	39	49.4	
4	12	10.5	10	28.5	2	2.5	

* χ^2 -test for difference in deceased and censored patients.[†] *t*-test for difference in deceased and censored patients.

GSTM1 and *GSTT1* were categorized as nonnull (reference) or null genotype.

Survival time was defined as time from beginning radiation treatment to date of death or date of last contact. Crude associations between genotypes and overall survival were assessed using Kaplan-Meier (KM) survival function. Test for differences of the survivorship functions by genotype was performed by means of log-rank test. Cox proportional hazards regression was additionally applied to calculate hazard ratios (HRs) and corresponding 95% confidence intervals (CI), adjusting for study, sex, and cancer stage. Age and additional treatment with chemotherapy did not affect HRs and were therefore not included in the model. Test for trend was performed by using the Wald statistic. Because a small proportion of patients died from other ($n = 3$) or unknown ($n = 2$) causes, we also conducted Cox proportional hazards regression analyses including only those who died from rectal cancer.

3. Results

All SNPs were in Hardy-Weinberg equilibrium. The observed allele frequencies of *GSTP1*, *CAT*, *MnSOD*, *MPO*, and *eNOS* were comparable to those reported for Caucasian populations in the dbSNP database. Frequencies of *GSTT1* and *GSTM1* deletion polymorphisms were in accordance with those previously reported [23].

Characteristics of the study population are presented in Table 1. No differences between deceased and censored

cases were observed with respect to sex, age, and BMI. As expected, notably more deceased patients suffered from advanced rectal cancer including stage 3 and stage 4 disease (82.8%) compared to censored patients (51.9%). Of the 114 patients who received radiotherapy, 39 individuals only received neoadjuvant treatment, 74 patients only received adjuvant/palliative treatment, and one patient received both (Table 2). Altogether, 31% ($n = 35$) of the patients died. Most deceased patients had stage 3 disease (54.3%).

Median followup time was 42.5 months. Plots of the estimated KM survivorship functions did not show differences in survival according to genotype for any of the polymorphisms (data not shown). However, multivariate Cox regression analyses showed a decreased hazard of death for patients carrying *MPO* G463A A allele compared to homozygous carriers of the G allele (HR: 0.44, 95% CI: 0.21–0.93; Table 3). This finding was consistent with borderline statistical significance of the log-rank test comparing KM survival curves by *MPO* genotype ($P_{\log\text{-rank}}$: 0.06). Patients heterozygous for the *MPO* A allele showed a HR of 0.39 (95% CI: 0.17–0.87), whereas patients homozygous for the A allele showed an HR of 1.12 (95% CI: 0.25–5.08) compared to homozygous carriers of the G allele. However, only seven patients were homozygous for *MPO* A allele.

Carrying the *eNOS* Glu²⁹⁸Asp asparagine allele was associated with a significantly increased hazard of death compared to homozygous carriers of the glutamine allele (HR: 2.10, 95% CI: 1.01–4.38; Table 3). Patients carrying one or two *eNOS* asparagine alleles showed an HR of 2.19 (95%

TABLE 2: Neoadjuvant and adjuvant/palliative radiation therapy.

	Neoadjuvant (N = 40)*		Adjuvant/Palliative (N = 75)*		Deceased (N = 35)	
	N	%	N	%	N	%
Stage 1	8	20.0	2	2.7	1	2.8
Stage 2	13	32.5	22	29.3	5	14.3
Stage 3	13	32.5	45	60.0	19	54.3
Stage 4	6	15.0	6	8.0	10	28.6

*One patient received both neoadjuvant and adjuvant/palliative radiation therapy.

TABLE 3: Hazard ratios of overall mortality in rectal cancer patients who received radiation therapy according to polymorphisms in *GSTP1*, *GSTT1*, *GSTM1*, *CAT*, *MnSOD*, *MPO*, and *eNOS*.

Genetic polymorphism	N Cases/Deaths	Person-years	Death/Person-years	$P_{\log\text{-rank}}$	HR [§]	(95% CI)	P_{trend}
<i>GSTP1</i>							
Ile/Ile	54/16	194.5	0.08		1		
Ile/Val	51/17	174.3	0.10		1.18	(0.58–2.40)	
Val/Val	9/2	33.0	0.06	.75	0.85	(0.19–3.85)	.88
Val carriers					1.14	(0.57–2.28)	
<i>GSTM1</i>							
nonnull	61/16	218.0	0.07		1		
null	53/19	183.8	0.10	.35	1.64	(0.84–3.21)	.15
<i>GSTT1</i>							
nonnull	91/31	321.5	0.10		1		
null	23/4	80.3	0.05	.17	0.68	(0.23–2.00)	.49
<i>CAT</i>							
C/C	65/20	225.2	0.09		1		
C/T	43/13	156.4	0.08		1.37	(0.65–2.87)	
T/T	6/2	20.1	0.10	.96	0.58	(0.13–2.71)	.98
T carriers					1.16	(0.58–2.30)	
<i>MnSOD</i>							
Val/Val	25/8	96.6	0.08		1		
Val/Ala	67/20	230.7	0.09		0.76	(0.31–1.85)	
Ala/Ala	20/7	68.5	0.10	.93	0.83	(0.27–2.51)	.75
Ala carriers					0.78	(0.33–1.84)	
<i>MPO</i>							
G/G	64/25	207.2	0.12		1		
G/A	43/8	172.2	0.05		0.39	(0.17–0.87)	
A/A	7/2	22.5	0.09	.06	1.12	(0.25–5.08)	.09
A carriers					0.44	(0.21–0.93)	
<i>eNOS</i>							
Glu/Glu	51/12	194.7	0.06		1		
Glu/Asp	53/20	175.5	0.11		2.19	(1.04–4.61)	
Asp/Asp	10/3	31.7	0.09	.29	1.56	(0.41–6.01)	.12
Asp carriers					2.10	(1.01–4.38)	

[§] Cox proportional hazards model adjusted for sex, study, and cancer stage.

CI: 1.04–4.61) and 1.56 (95% CI: 0.41–6.01), respectively, as compared to patients homozygous for the glutamine allele.

None of the other polymorphisms under study were found to be significantly associated with survival in rectal cancer patients who received radiotherapy. Associations between polymorphisms and rectal cancer-specific survival were similar to those investigating overall survival (data not shown).

4. Discussion

This study is the first explorative analysis to evaluate the effect of genetic polymorphisms in genes related to oxidative stress mechanisms on survival of rectal cancer patients after radiotherapy. Our data provide first suggestive evidence of an association between genetic variants and survival in rectal cancer patients who received ROS producing radiation

therapy. Due to genetic variation, higher amounts of ROS reaching tumor cells and exaggerating cytotoxic activity may increase survival. Since radiotherapy exerts part of its antineoplastic effect via the generation of oxidative stress, we assessed whether genotypes related to high levels of ROS may prolong survival in patients who received radiotherapy. As hypothesized, patients carrying *eNOS* Glu²⁹⁸Asp low ROS producing asparagine allele showed increased hazard of death as compared to noncarriers. This finding is biologically plausible as the *eNOS* asparagine allele has been related to low ROS levels [18]. Our finding is in line with a study in 873 breast cancer patients that observed a shorter survival in patients carrying the *eNOS* asparagine allele [19]. However, that study did not provide information on type of treatment that the patients received. Treatment and clinical course of rectal cancer and breast cancer are completely different; therefore comparison of results may not be useful.

Carrying low ROS producing *MPO* G463A A allele was associated with reduced hazard of death compared to noncarriers, but there was no allele dose effect. This observation lacks biological plausibility as the *MPO* G allele has been suggested to be associated with high levels of ROS, and genotypes related to high levels of oxidative stress have been proposed to prolong survival [10, 15]. A study of breast cancer patients indeed observed a survival benefit for patients homozygous for the *MPO* G allele who received treatment with chemo- and/or radiotherapy [10]. Nonetheless, our study showed a nonsignificantly increased HR for patients homozygous for the A allele based on a small number of patients.

Since our study is limited in size and a large number of associations were tested, chance findings with regard to the effect of *eNOS* and *MPO* polymorphisms on survival cannot be ruled out. However, recall bias could not have occurred in this study as we collected information about treatment modalities from the treating physicians. Nevertheless, it cannot be excluded that the treatment data were more likely provided on patients who were still alive or recently deceased causing selection bias.

Despite biological evidence that radiation exerts part of its effect via the generation of oxidative stress, the effect of genetically determined differences in oxidative stress mechanisms on survival after radiotherapy is not well understood. Further, larger studies providing detailed information about duration and intensity of radiation treatment are required to clarify the association of *eNOS* Glu²⁹⁸Asp and *MPO* G463A polymorphisms with survival in rectal cancer patients who received radiotherapy. Altogether, our findings highlight the need for further research on putative genetic markers in rectal cancer patients treated with radiotherapy.

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